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Crystallization of Membrane Proteins from Media Composed of Connected-Bilayer Gels

Abstract: *The use of hydrated-lipid gels in which the bilayer is an infinitely periodic (or at least continuous), three-dimensional structure offers a relatively new approach for the crystallization of membrane proteins. While excellent crystals of the Halobacterial rhodopsins have been obtained with such media, success remains poor in extending their use to other membrane proteins. Experience with crystallization of bacteriorhodopsin has led us to recognize a number of improvements that can be made in the use of such hydrated-gel media, which may now prove to be of general*

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value for the crystallization of other membrane proteins.
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INTRODUCTION

The phrase "structural genomics of membrane proteins" remains—at this time —no more than a combination of incongruous words; it is a phrase that reflects a highly sought-after goal, but the goal is one for which there is not yet a clear path to a practical realization. The difficulty of growing well-diffracting crystals is perhaps foremost among the problems that stand in the way of any effort to use high-throughput technologies to characterize the structures of membrane proteins on a whole-genome level. Until new methods are found that work as reliably as do the methods that are used for soluble proteins, structural studies of membrane proteins will surely continue to lag far behind those of the rest of the genome.

The use of existing methods for the crystallization of membrane proteins has nevertheless produced a significant number of high-resolution structures over the past decade. Membrane-protein structures with new functions are now reported at the rate of about 5 or more per year rather than one every 2–3 years. Furthermore, structures are now known for some membrane proteins of extraordinary interest, such as ion channels,[1–3] rhodopsin,[4] ABC transporters,[5,6] and a P-type ATPase.[7] Much more progress is still needed, however, if the study of membrane proteins is to be elevated to the level of "structural genomics."

The need to improve the rate at which it is possible to crystallize membrane proteins is especially acute when it comes to understanding the biochemical action of numerous drugs. Many aspects of cell biology and human physiology involve biochemical pathways and networks that begin with membrane proteins. Many of these membrane proteins, in turn, are targets for medical intervention as well as targets for drugs of abuse. These include chemically gated ion channels, receptors that mediate transmembrane signaling, and membrane transporters that move bioactive compounds across the cell membrane. Until methods are developed that can speed the structure determination for all of these diverse systems, current research must work under the severe handicap of not knowing the chemical details of ligand binding, or the molecular mechanism of the relevant protein function.

Broad success has recently been achieved in using lipid-bilayer gels to crystallize the Halobacterial family of rhodopsins.[8–13] Since this is a family of membrane proteins that had previously resisted crystallization, this success immediately raised the question whether such gels might not have equally high potential for use with other membrane proteins. However, only very limited success has been reported in crystallizing membrane proteins other than the Halobacterial rhodopsins.[14]

The failure to crystallize other membrane proteins as easily as one can crystallize the Halobacterial rhodopsins is in some sense quite surprising. Bacteriorhodopsin has been one of the more difficult membrane proteins to crystallize in the detergent-soluble state,[15] and thus one might have also thought that it would not be a promising protein to crystallize from lipid-bilayer gels. It seems unlikely that the admittedly "special" robustness of this protein is what accounts for its ability to crystallize from lipid-bilayer gels, since that same robustness gives bacteriorhodopsin no preferred advantage for crystallization in the detergent-solubilized state.

Nevertheless, some clues to ways in which crystallization from lipid-bilayer gels might be applied more broadly are beginning to emerge from the growing number of crystallization screens that have been carried out with the Halobacterial rhodopsins. Successful searches have been completed for conditions to crystallize not only proteins homologous to bacteriorhodopsin—i.e., halorhodopsin[10] and sensory rhodopsin II[11,12]—but also a number of mutants of bacteriorhodopsin. As is summarized in Table I, these proteins crystallize in multiple space groups and in a variety of mother-liquor solutions.

A number of specific conclusions and recommendations can be formulated from these studies on Halobacterial rhodopsins, which can next be tested with a broader range of membrane proteins. Foremost among our recommendations is that membrane lipids should be included when crystallizing membrane proteins from a lipid-bilayer gel. The dimensions of parameter space explored in crystallization trials should be expanded to cover both the amount and the chemical type of "guest lipid" that is added to the bilayer gel. A second recommendation is to screen other gel-forming lipids in addition to mono-olein as the host bilayer. Since it is likely that a much larger number of parameters will have to be explored when

Table I Representative Examples of Successful Crystallization of Halobacterial Rhodopsins from Connected-Bilayer Gels

Type of Protein	wt bR[9,44]	wt bR[13]	hR[10]	sR II[1] Halobacterium	sR II[12] Natrono-bacterium	bR[45] D85S Mutant	bR* F219L Mutant	bR[46] D96N Mutant	bR[47] E204Q Mutant
Aqueous precipitant	≈3*M* phosphate	High phosphate (bicelle "hanging drop")	4*M* KCl 50 m*M* Tris	3.5*M* KCl 50 m*M* MES	75 m*M* NaOAc 1.5*M* NaCl	25 m*M* phosphate	≈3*M* phosphate	≈3*M* phosphate	≈3*M* phosphate
Space group	$P6_3$	$P2_1$	$P6_322$	$C222_1$	$C222_1$	$C222_1$	$P6_3$	$P6_3$	$P6_3$
Resolution (Å)	≈1.6	≈2.0	≈1.8	≈2.4	≈2.1	≈2.3	≈1.8	≈1.8	≈1.7

[a]Unpublished (Rouhani et al.).

crystallizing membrane proteins from lipid-bilayer gels, it will be important to develop technologies that allow higher throughput while screening, and at the same time allow the use of smaller amounts of protein for each condition that is being tested. A final point is that membrane proteins cannot be expected to be more stable in the bilayer gel than they are in the detergent-solubilized state, contrary to what one might reasonably have expected would be the case.

CRYSTALLIZATION FROM A MEDIUM OF CONNECTED LIPID-BILAYERS INVOLVES SOME NOVEL PHYSICAL AND CHEMICAL PRINCIPLES

The stable, hydrated gel that is formed by mono-olein is in effect a single lipid bilayer, curved in the shape of a saddle at every point on its surface. As one might well imagine, such a surface will soon run into itself in three dimensions. The result is, indeed, the formation of a three-dimensionally connected, hydrated bilayer, a structure that we hereafter refer to as a *connected-bilayer gel* (CBG).

The idea that a lipid bilayer can form a three-dimensionally connected structure is one that is perhaps not intuitively obvious or easily visualized. The difficulty, at least initially, in visualizing bilayers that extend indefinitely in three dimensions is probably due to the usual "textbook" representation of a bilayer as an infinite plane or as a spherical, closed shell (a liposome). One example of a three-dimensionally connected bilayer that can be quite easily imagined, however, consists of close-packed vesicles between each of which a "fusion neck" has been formed to every neighbor with which it is in contact. It is easy to see that such a three-dimensionally connected bilayer would form a relatively stiff gel, since the membrane pieces could no longer flow relative to one another as would be possible for planar sheets or spherical liposomes. One can speculate that precisely this type of connected-bilayer gel was formed by dehydration of proteoliposomes containing bacteriorhodopsin, from which three-dimensional protein crystals were obtained by Takeda et al.[16]

The example of a CBG that is arguably the best understood in structural terms is the $p\bar{3}nm$ "cubic phase gel" that is formed by hydrated mono-olein (MO).[17] This gel is formed by the assembly of tetrahedral units, whose structure is sketched in the cartoon that is shown in Figure 1. These units are then bonded together to form the so-called double diamond or D cubic phase. Harper and Gruner[18] give an informative review of the historical discovery and mathe-

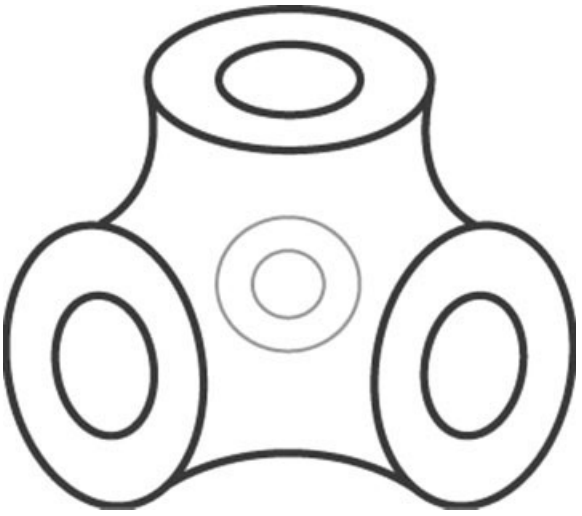


FIGURE 1 Cartoon representation of the fundamental building block from which the pn$\bar{3}$m cubic phase of the hydrated, connected-bilayer gel of mono-olein is constructed. The fundamental building block is a tetrahedral unit whose faces are circular cross-sections through a three-dimensionally continuous lipid bilayer. The gel itself is built by docking the faces of adjacent tetrahedra together to create a lattice that extends indefinitely in three dimensions. The pn$\bar{3}$m cubic phase represents a gel in which these tetrahedra are bonded together in the so-called double-diamond lattice.

matical characterization of the three types of "infinite periodic minimal surfaces" that are the basis for the cubic phases that can be formed by hydrated mono-olein. In the case of mono-olein, a monoglyceride, the pn$\bar{3}$m gel has the extremely useful property that it coexists in equilibrium with excess water. The composition-temperature phase diagram depicting the conditions under which this and other phases are thermodynamically stable has been determined with particularly great accuracy for mono-olein.[17]

There are, however, a number of other lipids that make ordered CBGs. A variety of monoglycerides, in addition to mono-olein, are known to make connected-bilayer gels.[19] Diacylphosphatidylethanolamines are a second class of lipids that form isotropic gels when hydrated.[20–26] It is noteworthy that there seems to be a connection between the ability of lipids to form CBGs and their ability to induce membrane fusion,[27,28] a point that is reminiscent of the picture used above to visualize how close-packed liposomes could be converted, by fusion, into a connected-bilayer gel.

While the cartoon representation of the tetrahedral unit that is shown in Figure 1 is helpful for visualizing how a lipid bilayer can extend continuously in three dimensions, it is by no means necessary that connected bilayer gels form periodic or repeating structures. Tetrahedral building blocks can also be assembled to produce totally random networks (amorphous ice; amorphous silicon) rather than crystalline solids. In these examples, however, the amorphous solid is metastable with respect to one or another ordered phase, and perhaps the same must be true for the corresponding CBG systems.[29]

Insertion of a membrane protein into a CBG provides two elements of what could prove to be quite generally favorable conditions for crystallization. A lipid bilayer is, of course, the natural environment for a membrane protein, and thus one would expect that all membrane proteins might be stable once they have been reconstituted into such gels, albeit this is not always the case. Second, proteins that are inserted into a CBG are able to diffuse in three dimensions, almost as freely as when they are dissolved in an aqueous buffer (in the detergent-solubilized state). Inserted membrane proteins are thus able to collide with one another, nucleate, and add to the edges and faces of growing crystals.

The use of CBGs to crystallize membrane proteins thus requires only three elements.

1. The membrane protein must insert itself into the membranes of the CBG. In current practice, this is accomplished simultaneously with formation of the hydrated gel itself, as is described in the following section. The driving force for insertion could well be removal of detergent from the aqueous phase, and thus from the solubilized membrane protein itself, since the detergent itself is likely to partition into the lipid bilayers. Alternatively, bacteriorhodopsin can be inserted into the hydrated MO gel by simply mixing unsolubilized purple membrane with the gel,[30] a fact that implies that the MO bilayers are able to fuse to the rims of the purple membrane. From this example, it follows that membrane proteins could also be reconstituted first into proteoliposomes, and these could be mixed with the CBG, releasing the protein into the bilayers of the gel by fusion with the liposomes.
2. The lipids that make up the CBG must be fluid. Fluidity is needed, of course, to allow the proteins to diffuse freely in space, as mentioned above.
3. There must be a thermodynamic driving force that tends to expel the inserted membrane proteins from the bilayer gel, so that they will join a growing crystal once there has been a nucleation event. The primary driving force can be favorable protein–protein contacts, of course, just as is the case for the crystallization of

soluble proteins. In most cases the enthalpy of protein–protein contacts is made favorable by the addition of suitable buffers or precipitants. It is also conceivable that the protein-induced perturbation of the "minimal energy surface" formed by the lipid (the protein being in effect an impurity within the CBG) may also contribute to the driving force that tends to expel the protein. More generally, any thermodynamic effect that leads to lateral phase separation—and even better, to two-dimensional crystallization of the membrane protein—will also be favorable for the formation of three-dimensional protein crystals.

Crystallization from a CBG medium thus involves principles that are, at least in part, fundamentally different from previous strategies for crystallization of membrane proteins. The "traditional" method, pioneered by Michel,[31] treats detergent-solubilized membrane proteins in essentially the same way as any other soluble macromolecule. As is indicated schematically in Figure 2a, precipitant solutions are mixed with the solubilized protein in screens that can be set up either as sitting drops or hanging drops. In general, it is considered advisable to avoid the use of membrane lipids as one of the components in such a crystallization screen.

Two-dimensional crystals, on the other hand, are grown by a variety of methods,[32–38] shown schematically in Figure 2b, in which detergent-solubilized lipid is added to the detergent-solubilized membrane protein, and then the detergent is removed by dialysis or by adsorption to polystyrene divinylbenzene beads (Bio-Beads, BioRad Laboratories, Hercules, CA).

The growth of three-dimensional (3D) crystals from CBGs, on the other hand, first involves removal of detergent by dilution into the CBG, which occurs at the same time as the protein is "reconstituted" into the bilayers making up the gel. Crystallization is then induced by addition of precipitants, as in the traditional method of 3D crystallization. The process of crystallization from a CBG medium, indicated schematically in Figure 2c, may actually be quite closely related to that by which two-dimensional (2D) crystals of membrane proteins are grown for electron diffraction and electron microscopy. One aspect that the two methods clearly have in common is the fact that detergent is removed, either by dialysis or by dilution, before crystallization occurs. A second point of similarity lies in the fact that crystals grown from CBGs, at least to date, all consist of coherently stacked sheets of 2D crystals. The only real difference between the two may be that successive 2D crystalline layers nucleate more easily when growth occurs within the gel, whereas this is something that occurs only rarely for most membrane proteins, when detergent is removed by dialysis.

The similarities that are likely to exist between true, single-layer 2D crystallization and the growth of stacked "2D crystals" from CBG media suggest that it may be essential to include membrane lipids when formulating the crystallization trials. Membrane lipids, rather than the CBG-forming lipid itself, are likely to be needed to serve as a "mortar" to fill small gaps and spaces between the hydrophobic surfaces of membrane-protein "bricks," as the latter assemble into 2D crystals (or 2D layers within a 3D crystal). A certain amount of lipid must be added, often in a mass ratio of 1:1 lipid:protein in order to produce 2D crystals upon removal of detergent.[32–35] Considerably higher ratios of lipid:protein are likely to be required when growing 3D crystals from CBGs, however. Added lipid has nowhere else to go, other than with the reconstituted protein, when detergent is removed by dialysis. In the CBG system, on the other hand, the entropy of dilution into the CBG will oppose the recruitment of added lipid to the growing crystal, an effect that can nevertheless be offset by adding a much higher ratio of lipid relative to protein.

The opportunity—even the necessity to include either natural or artificial membrane lipids in the crystallization protocol—is expected to be one of the advantages of using the CBG medium. Unlike the situation of the "traditional" crystallization of detergent-solubilized membrane proteins, membrane lipids can—even should be added to the protein immediately after purification. Lipids can even be included during all stages of solubilization and purification. This fact can potentially be a great advantage, since it is well known that added lipids greatly stabilize detergent-solubilized membrane proteins. On the other hand, we discuss later an example in which a bacteriorhodopsin mutant was actually destabilized by adding purple membrane lipids to an MO gel.

It also seems likely that the use of CBG media may allow a much wider range of detergents to be used for protein solubilization and purification. By the very nature of the process, the initial detergent belt is removed from the reconstituted protein and becomes irrelevant as crystallization proceeds. This fact means that one may have greater freedom to choose a detergent that best stabilizes the protein during purification, without being concerned that the detergent will subsequently cause steric interference that prevents formation of stable protein–protein contacts. Furthermore, unlike the case of 2D crystallization, one may

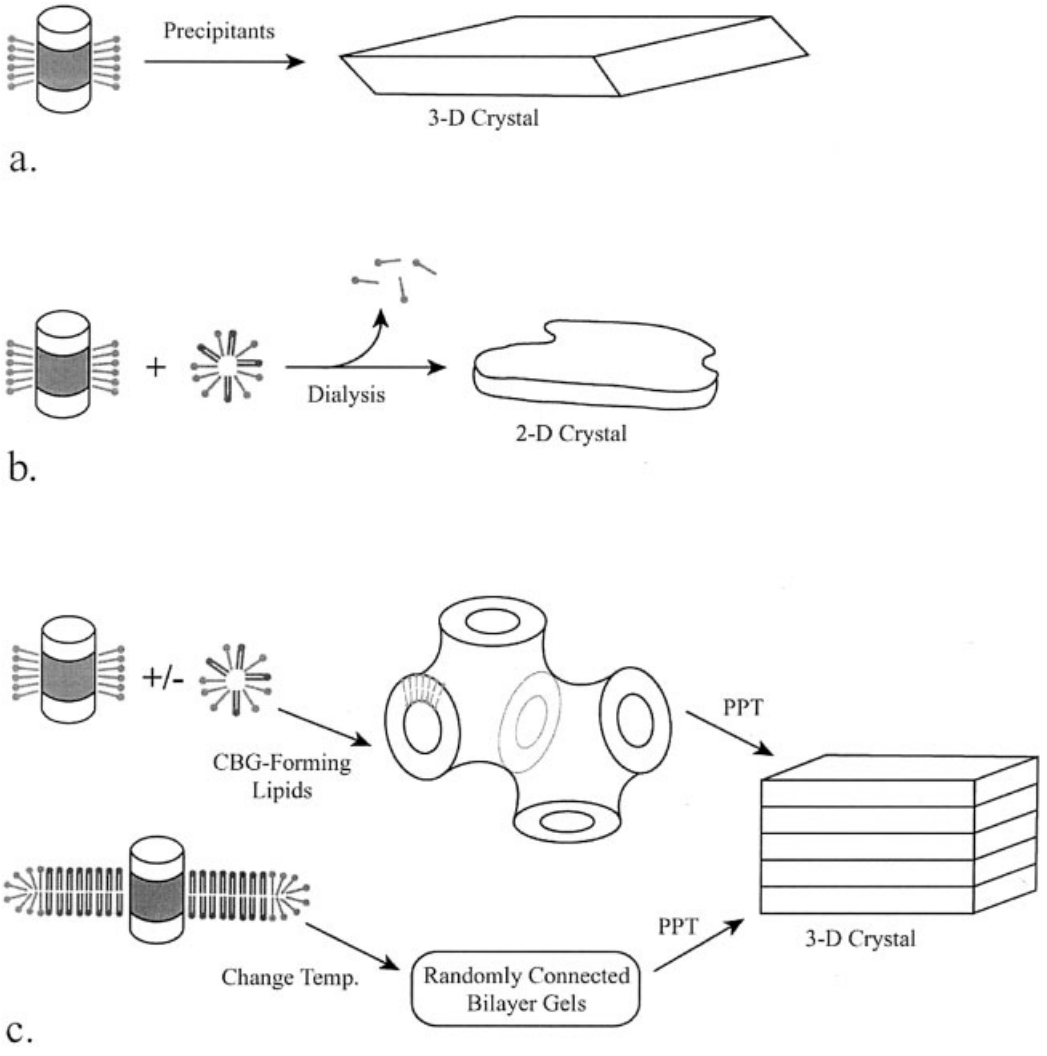


FIGURE 2 Schematic diagrams that illustrate the processes involved in crystallization of membrane proteins. (a) The conventional process that is used to obtain three-dimensional crystals is one in which the detergent-solubilized membrane protein is handled in the same way that one would crystallize any soluble macromolecule. It is normally thought that membrane lipids should not be added to the crystallization setups in this case. (b) Two-dimensional crystals of detergent-solubilized membrane proteins, suitable for electron diffraction experiments, are normally obtained by adding excess membrane lipids and then removing the detergent by dialysis, or equivalently, by the use of Bio-beads (Biorad Laboratories, Hercules, CA). At a high protein:lipid ratio the protein and lipid spontaneously assemble into bilayers in which the protein density is so high that formation of a 2D crystalline lattice can occur quite easily. (c) Growth of three-dimensional crystals from a connected-bilayer gel (CBG) involves a process of "reconstitution" of the detergent-solubilized membrane protein, not unlike that which happens when detergent is removed by dialysis in the presence of excess lipid. In this case, however, detergent is removed by a process of partitioning into the CBG. Crystallization of the reconstituted membrane protein is then induced by addition of buffers and precipitants that favor the formation of protein–protein contacts, just as in the case of the conventional process shown in panel (a).

no longer need to be concerned whether the detergent can be easily removed by dialysis.

In closing this section, it is worth emphasizing that the creation of an ordered lattice of connected lipid bilayers is not the only way in which to form a CBG, nor are the fusogenic lipids the only ones that are able to form CBGs. In a novel approach developed recently by Faham and Bowie,[13] bacteriorhodopsin was

first reconstituted into CHAPSO-stabilized lipid-bilayer disks (bicelles). These bilayer disks were then fused into a CBG by raising the temperature from 0 to 37°C. Crystallization of bacteriorhodopsin followed, once a pathway for 3D diffusion of the membrane protein was established. As was mentioned previously, dehydration of densely packed proteoliposomes[16] may represent yet another route to the successful formation of CBGs, from which membrane proteins can be crystallized under suitable buffer conditions.

CRYSTALLIZATION OF THE HALOBACTERIAL RHODOPSINS HAS PROVIDED A BASIC FOUNDATION FOR BROADER APPLICATIONS OF THE TECHNIQUE

Halobacterial Rhodopsins Crystallize Under a Variety of Conditions and in a Variety of Space Groups

Crystallization of the Halobacterial rhodopsins from the pn$\bar{3}$m cubic phase of mono-olein has been successful under quite varied conditions. Table I is a partial list of the types of crystals that have been obtained for this family of proteins, all of them diffracting at high resolution. The added precipitants have ranged from 3*M* phosphate or 4*M* KCl to 25 m*M* phosphate. Crystals have grown in the orthorhombic and monoclinic space groups as well as in two different space groups within the hexagonal group. One feature that is held in common is the fact that all crystals consist of coherently stacked membrane sheets. The protein and lipid molecules are arranged as a two-dimensional crystal within each sheet, and protein–protein contacts are made between the hydrophilic surfaces of the membrane proteins in successive layers.

Crystallization from Connected-Bilayer Gels is Compatible with only a Limited Range of Additives

While the use of CBGs should, in theory, allow an unlimited choice for the type of detergent that is used during solubilization and purification, in practice this choice may still be quite restricted. Even when the CBG itself will tolerate the detergent, it is not necessarily the case that crystals of the membrane protein will be obtained under otherwise identical conditions. Our own experience, for example, is that bacteriorhodopsin did not crystallize from the MO CBG in 3*M* phosphate buffer when the protein was solubilized by Triton detergent, even though the protein remained stable (purple) in the MO gel.

As is to be expected, a CBG can tolerate only a limited amount of detergent that can be present before the lipid is solubilized. Well before that point, however, the MO CBG is observed to convert to a fluid, bilayer phase, and after that an oily separation occurs as more detergent is added. As a rule of thumb, CBGs may tolerate only 0.5–1.5% detergent by weight, depending upon the detergent that is being used.[19]

Similarly, while the use of CBG media may allow, and even require the addition of membrane lipids, the chemical type of such lipids—as well as the amount of such lipids that can be used—may again be somewhat limited. As a rule of thumb, the MO CBG will tolerate 5–10% "guest" lipid, and in some cases even more.[19] We have found that the pn$\bar{3}$m gel of MO will easily tolerate 4% lipid that has been extracted from purple membrane by the method of Kates et al.[39] However, as little as a 1:1 ratio of added lipid relative to solubilized purple membrane was found to destabilize (and ultimately bleach) a particular mutant of bacteriorhodopsin.

CBGs often have a limited range of temperature over which they can be used. As is shown clearly in the published phase diagram for the MO–water system,[17] the pn$\bar{3}$m cubic phase is not stable below ~18°C. In accord with this phase diagram, we find that both optically clear lipid–water gels and purple, optically clear crystallization setups of bacteriorhodopsin became turbid within hours of cooling to 4°C. Hydrated gels and crystallization setups formed with monopalmitin (MP), on the other hand, remain clear when cooled to 4°C. Even so, there is still a risk that the MP gel may become cloudy when certain precipitants are overlaid at low temperature, an effect that we have observed with 3*M* phosphate buffer. The CBG system formed from bicelles[13] is currently unable to work at low temperature, however, since satisfactory gel formation occurs only upon raising the temperature above 37°C.

The stability of CBGs can also be affected by aqueous-phase precipitants that are used to induce protein crystallization. Experience in this regard is limited primarily to the fully hydrated MO cubic phase. Since the pn$\bar{3}$m phase of this monoglyceride is stable in the presence of excess water, aliquots of standard protein-crystallization media can be overlaid on small aliquots of gel to determine whether the gel remains stiff, optically clear and optically isotropic (i.e., nonbirefringent).[40,41] In our own tests of this type, we screened 160 nonredundant solutions from 4 Hampton (Laguna Niguel, CA) kits (crystal screen, crystal screen 2, Membfac, and low ionic strength).

Of these, 112 proved to be compatible with maintaining the original properties of the gel. As a general rule, high concentrations of alcohols including 2-methyl-2,4-pentanediol (MPD), and high molecular weight polyethylene glycols (PEGs) are incompatible with formation of the desired gel.

Halobacterial Rhodopsins Are Not Necessarily as Stable in a Bilayer Gel as They Are in the Detergent-Solubilized State

The assumption that a membrane protein will automatically be more stable in the CBG than it is in detergent has been found to be false for two of the bacteriorhodopsin mutants with which we have been working. Our first experience in this regard occurred during crystallization of the F219L mutant of bacteriorhodopsin. The F219L mutant is itself relatively unstable when solubilized in octylglycoside, and it proved to be even more unstable when reconstituted into the $pn\bar{3}m$ cubic phase of MO. The addition of excess retinal was found to stabilize the protein after reconstitution in MO, however, leading to successful crystallization under conditions similar to those used for wild-type bacteriorhodopsin.

The stability of the protein, once it has been reconstituted within the CBG, can be affected either favorably or unfavorably by the choice of the aqueous-phase precipitant. Of the 112 Hampton solutions that appear to be compatible with maintaining the cubic phase of MO, for example, more than half cause wild-type bacteriorhodopsin (bR) to either bleach or markedly change its color.

The Type and Quality of Crystals of bRs That Are Obtained Depend Upon the Type of CBG That is Used and the Temperature of Crystallization

As was already noted in the first paper of Landau and Rosenbusch,[8] wild-type (wt) bacteriorhodopsin crystallizes from MO as hexagonal plates at room temperature, but as thin rhomboid crystals from MP. The rhombic crystals were reported to diffract rather poorly, and our own experience has been that they may be poorly shaped, even lacking any apparent crystal edges. As a result, we had long considered it to be unwise to attempt further crystallization screens in MP. Nevertheless, we have recently found that unusually large, well-formed crystals of wild-type bacteriorhodopsin are obtained (Figure 3), which diffract to ~2 Å resolution (Figure 4) when setups of wt bR in MP are incubated at 4°C. We have also had success in crystallizing wt bR from MP as small hexagonal plates (Figure 5) at room temperature, provided that additional purple membrane lipids are included as part of the setup. These small hexagonal crystals also diffract to ~2 Å resolution. On the basis of these results, we would now recommend that as much emphasis should be given to crystallization from MP as from MO, especially since the MP gels can support crystallization at low temperatures.

FIGURE 3 Micrograph of a large rhombic crystal of wild-type bacteriorhodopsin that was grown from a hydrated CBG of monopalmitin. Although only poor crystals are formed in this system when incubated at room temperature, large and well-formed crystals can be grown by incubating the setups at 4°C.

The addition of purple membrane lipid can have a significant effect on the outcome of screening trials. Unfractionated lipid, extracted from purple membrane by the method of Kates et al.,[39] greatly accelerates the formation of "mature-sized" crystals of wild-type bR in the MO CBG, as is documented by the results shown in Figure 6. However, the limiting size of the crystals produced in setups with added lipid is no larger than that obtained without extra lipid. Furthermore, we found no improvement in the resolution limit of the diffraction data, no difference in the crystalline lattice constants, and

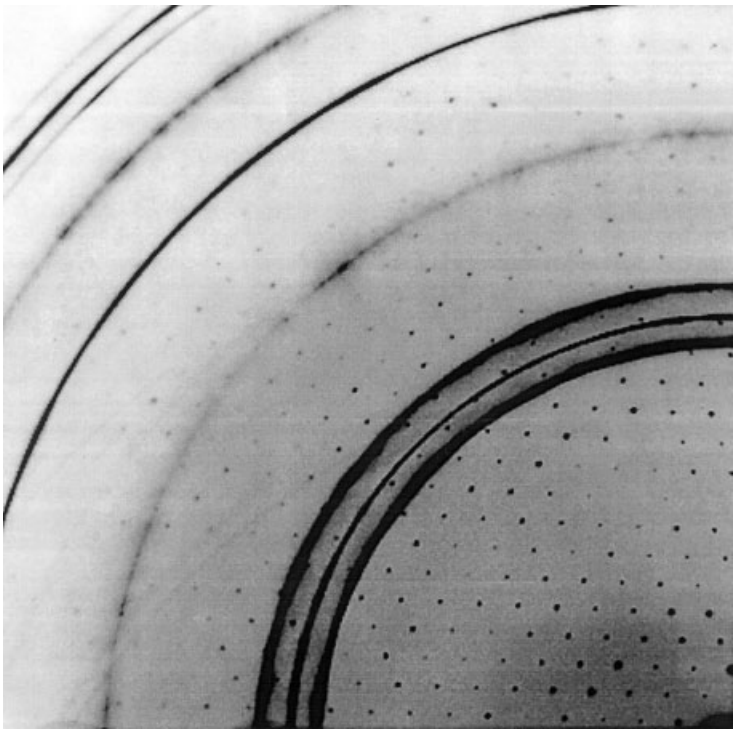


FIGURE 4 X-ray diffraction pattern obtained from a rhombic crystal of wild-type bacteriorhodopsin like the one shown in Figure 3. The diffraction pattern extends beyond a resolution of 2.2 Å.

no decrease in the tendency to produce twinned crystals with nearly equal twin fractions. Added lipid did influence the crystal type that could be grown from MP at room temperature, however, although not at 4°C.

The use of other lipids that form CBGs still remains largely to be investigated. As mentioned previously, phosphatidylethanolamines (PEs), as well as monoglycerides (MGs), are already known to make isotropic cubic phases when hydrated. CBGs formed

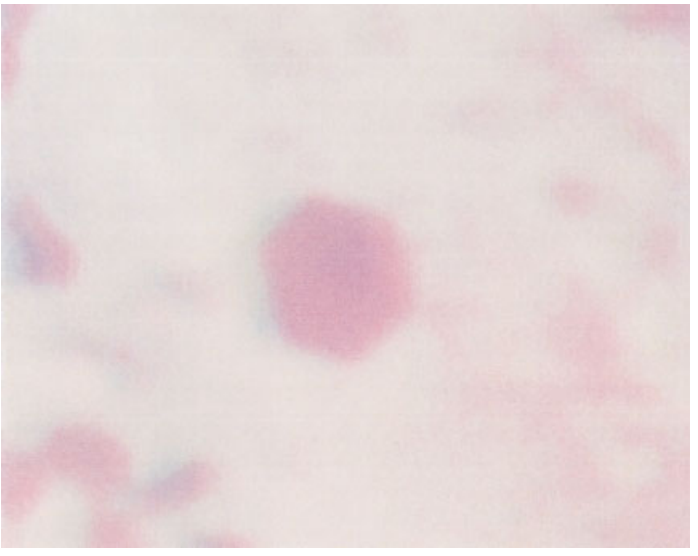


FIGURE 5 Micrograph of a well-formed hexagonal crystal of wild-type bacteriorhodopsin that was grown at room temperature from a hydrated CBG of monopalmitin. In this case excess purple-membrane lipid was added together with detergent-solubilized purple membrane. Although these crystals are smaller than those grown in mono-olein gels, x-ray diffraction patterns nevertheless extend to at least 2.3 Å resolution (data not shown).

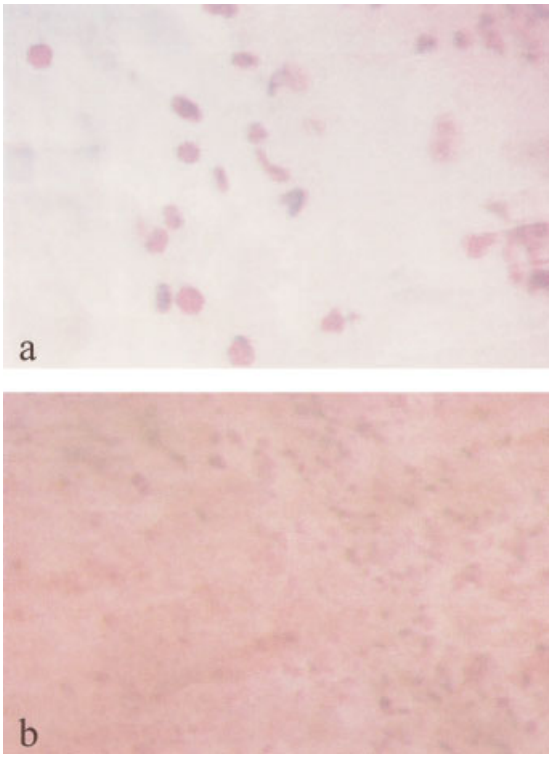


FIGURE 6 Demonstration that crystal growth can be greatly accelerated by the addition of excess membrane lipid. Panels (a) and (b) show bacteriorhodopsin crystallization setups that were prepared at the same time, which are identical in all respects except for the fact that the setup shown in (a) included a 1:1 ratio of excess lipid:solubilized purple membrane, while (b) included only the solubilized purple membrane. Both photographs were recorded one week after setting up the crystallization experiment. The crystal size observed in (b) is typical of what we usually see at this "short" time, while the crystals seen in the tubes with excess lipid, panel (a), have already reached their mature size.

by PEs are for the most part less attractive as potential crystallization media than MGs, however, since they require rather extreme thermal cycling[21,24] or the inclusion of fusogenic additives[22,25] to form a well-equilibrated gel. However, we have found that a polyethyleneglycol derivative of PE (mPEG 550 PE; Avanti Polar Lipids, Inc., Alabaster, AL) is easily hydrated and forms a stiff gel that gives several orders of x-ray diffraction rings (Figure 7). A disadvantage of this system, however, is that it is not stable in the presence of excess water, and thus one cannot add salts or precipitants by simply overlaying the gel with test solutions from a kit. Furthermore, preliminary tests show that wt bR bleaches within 24 h after reconstitution into this system, even when excess retinal has been added. Further characterization of the PEG–PE system that is worthwhile to explore would include determining the compatibility of this system with higher levels of native-membrane lipids, which in turn may lead to a proper stabilization of the native conformation of most membrane proteins.

Detergent-Solubilized Membrane Protein Inserts Spontaneously into the CBG

Bacteriorhodopsin can be easily "reconstituted" into CBGs during hydration of the lipid. A commonly used protocol involves weighing 13–14 mg of finely divided powder of monoglyceride (MG) "wax" into small glass tubes (or alternatively, into PCR tubes). Detergent-solubilized protein is pipetted into each tube at the desired protein concentration, with or without desired salts or precipitants. The tube is then centrifuged (without prior mixing or stirring) in a fixed-angle rotor at approximately 10,000 $\times$ g. It is usual in this type of approach to rotate the tubes at 15-min (or longer) intervals in an effort to improve "uniform" mixing of protein within the gel. We normally use equal weights of MG and protein solution in order to ensure having a small excess of water, but we avoid adding too much aqueous solution as the MG will then merely float on top of the aqueous phase. Once formation of a hydrated gel has been accomplished, buffers and precipitants can be overlaid onto the gel, to allow the components to diffuse into the bicontinuous aqueous channels of the gel.

We normally do not observe bleaching of bacteriorhodopsin during the actual process of reconstituting the detergent-solubilized protein into the hydrated gel, even for systems in which bleaching may occur later (on the time scale of hours or longer). The general absence of bleaching during the initial setup argues that the protein is not put at acute risk as detergent partitions into the large reservoir of lipid bilayers, or as the protein itself partitions into the hydrated bilayers. In fact, surprisingly in our view, we have observed that the octyl-glucoside-solubilized D85S mutant of bR remains stable in the aqueous phase when it is not completely mixed with the MO gel. Some of this protein then diffuses into the gel and forms crystals at a considerable depth within the otherwise clear gel. This observation was facilitated by the fact that the buffer suitable for crystal formation happens to be, in this case, the same as that used for solubilizing the bR-containing membranes. The long-term stability of the detergent-solubilized bacteriorhodopsin within an aqueous phase, from which the protein and thus also the detergent clearly have access to the bilayer system of the gel, is likely to be due to reassembly of the membrane lipids with the mem-

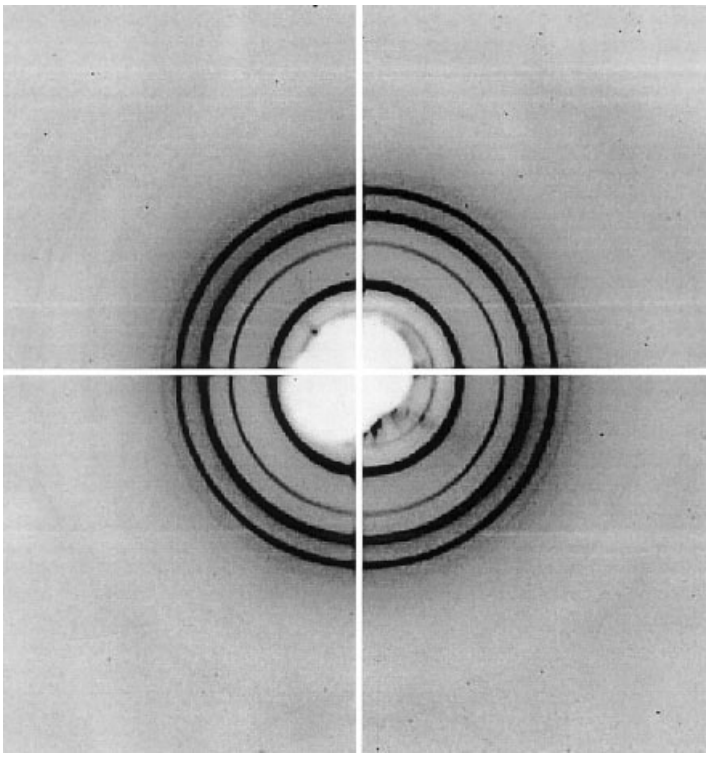


FIGURE 7 X-ray diffraction pattern obtained from a hydrated gel of PEG- derivatized DMPE; see text for details. The presence of several orders of diffraction rings demonstrates that this gel consists of a quite well-ordered lattice.

brane protein as detergent is depleted from the aqueous phase, just as is the case when detergent is removed by dialysis.

Bacteriorhodopsin can also be "reconstituted" into CBGs with the dual-syringe equipment that was developed in Caffrey's group to bring hydrated gels to thermodynamic equilibrium rapidly. Figure 8a is a photograph of two 250 μL Hamilton syringes and a custom-built coupler[42] by which the syringes can be connected in the head-to-head configuration that is shown in Figure 8b. (We thank Dr. Martin Caffrey for the gift of these couplers; similar devices can be purchased from Emerald Biostructures, Bainbridge Island, WA.) Prior to coupling, one syringe is loaded with a solution of detergent-solubilized protein and the other syringe is loaded with monoglyceride oil (obtained by melting the wax). After the preloaded syringes are coupled together, the hydrated CBG is formed by repeatedly pushing the components from one syringe to the other. Since the CBG is much more viscous than either of the component liquids, care must be taken to apply a force on the piston of the Hamilton syringe that is precisely on axis, to avoid fracture of the syringe. Mixing should also be performed in a slow, steady motion in order to prevent the development of turbidity within the gel during hydration of the lipid. We have had excellent success with this apparatus, using syringes that range from 50 μL to 1.0 mL in size. The syringe method is rapid (hydration is typically complete in 30 min) and produces a truly uniform distribution of protein within the gel.

Reconstitution of protein with the syringe apparatus also provides a good way to dispense very small aliquots for crystallization trials.[40] Once the hydrated gel has been formed, relatively large aliquots of gel can be transferred into a 10–250 μL Hamilton syringe so that much smaller aliquots, down to 0.2 μL in volume, can be dispensed with the ratchet device shown in Figure 8c (The Hamilton Company, Reno, NV).

Crystallization conditions can be screened by overlaying precipitant solutions onto the CBG.[40] Crystallization has conventionally been done in small glass or plastic tubes, but we now prefer to use 96-well plates, as was done by Royant et al.[12] In current practice, we use an 8-barrel pipettor to transfer 20 to 100 μL volume aliquots from a matrix of buffer and precipitant solutions. These overlay solutions are added to the wells as soon as possible after delivering aliquots of CBG, in order to avoid dehydration of the gel through evaporation. Once a full 96-well tray has

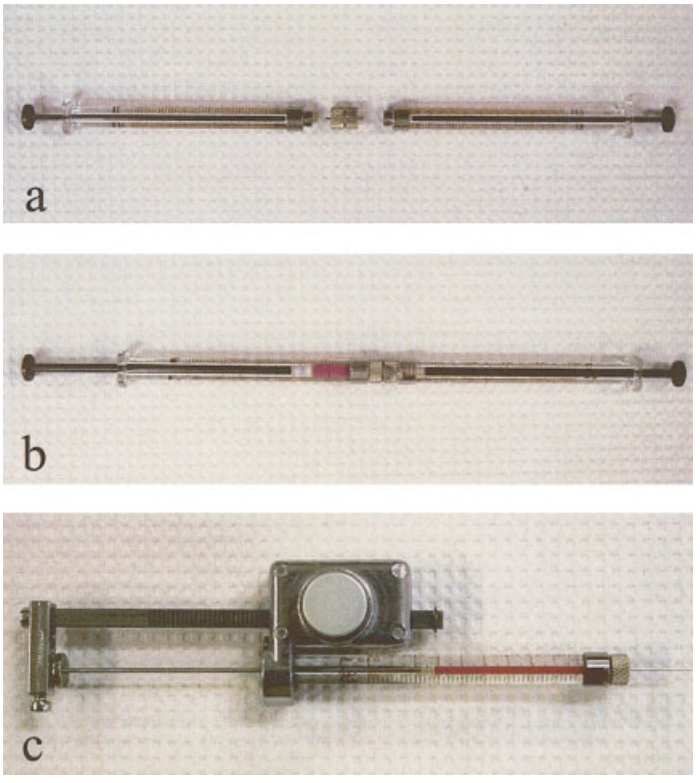


FIGURE 8 Apparatus that can be used to obtain a rapid and uniform reconstitution of detergent-solubilized protein into a hydrated CBG. (a) Two Hamilton syringes of equal size are used, one of which is filled no more than half way with the detergent-solubilized membrane protein, and the other of which is filled no more than half way with melted lipid. (b) The two syringes are then coupled with a watertight fitting that allows the repeated transfer of fluid from one syringe to the other, ultimately resulting in a uniform mixture of the two. (c) Relatively large aliquots of the hydrated gel can then be transferred into a third syringe, again using the coupling piece, so that small, precise aliquots can be delivered into tubes or the wells of a microtiter plate by the action of a mechanical ratchet.

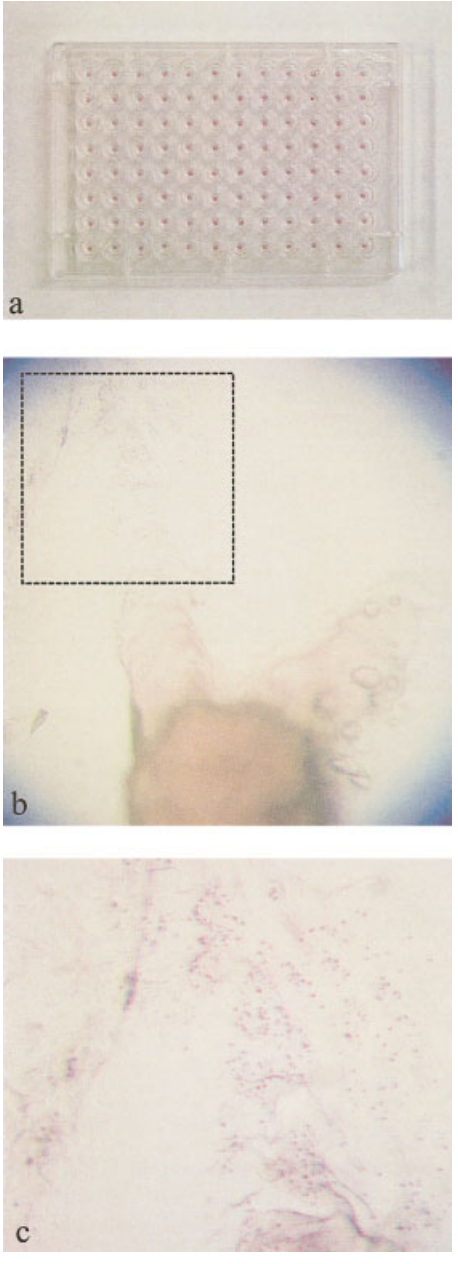
been set up, the wells are sealed with ClearSeal tape (Hampton, Laguna Niguel, CA). An example of the final result produced in one of these setups is shown in Figure 9a, while high-magnification images of the contents of one such well are shown in Figures 9b and 9c.

Crystal Growth Can Be Monitored by Conventional Light Microscopy

Polarization optics can be used to visualize microcrystals of uncolored proteins provided that screening is done with glass rather than plastic tubes. It should be noted, however, that such crystals are no more difficult to image by bright field optics when in a lipid gel than in water. The refractive index of the stiff gel, which will float on water, is actually less than that of water, thus allowing the same contrast mechanisms to operate in both cases. Finally, it is worthwhile to mention that polarization optics can serve as a useful quality control, to ensure that the CBC is itself not birefringent. To date, crystals of bacteriorhodopsin and its mutants have been obtained predominantly from optically isotropic gels, and we are inclined to recommend that birefringent gels are to be avoided.

Not all that glitters under polarization optics is a protein crystal, however. It is possible for mono-olein itself to grow relatively large clusters of thin, blade-like crystals. We have even observed this to occur spontaneously in gels consisting only of water and MO. More frequently, however, blades or laths, similar to the one shown in Figure 10, grow when there is protein in the setup but not in the buffer-only control. Crystalline material dissected from such gels, although "contaminated" with isotropic gel, runs as a single spot on thin layer chromatography (TLC), indistinguishable from reagent MO. TLC thus rules out the possibility that the crystalline laths are fatty acid

that has been produced by hydrolysis of MO, or a product of oxidation of the C9–C10 double bond in MO. X-ray diffraction gives photographs like the one shown in Figure 11, demonstrating that the lattice constants are too small for the crystals to have been produced by a protein or other macromolecule, but with one "large" lattice constant, consistent with the linear chain-length of MO. One remaining possibility, that the laths are co-crystals of MO isomers, remains still to be investigated.



DISCUSSION

The first question that must be asked at this point is why the use of CBG media has been so successful for crystallizing the Halobacterial rhodopsins, but so unsuccessful for other membrane proteins. It is true that there currently are three exceptions, i.e., proteins that are not in the family of Halobacterial rhodopsins, which have been crystallized from the mono-olein CBG.[14]

One factor that might be a unique advantage for the Halobacterial rhodopsins is that they all possess a very tightly bound (indeed, covalently bound) ligand, which nevertheless is easily released from the denatured form of the protein. Ligands that bind only to the native state shift the ratio of native to unfolded protein according to the equation

$$[N]/[U] = K_f(1 + [L]/K_d)$$

where $K_f = [N]/[U]$ in the absence of L, $[N]$ and $[U]$ are the concentrations of native and unfolded proteins, respectively, $[L]$ is the concentration of ligand and K_d is the dissociation constant for the ligand.[43] Ligand binding can thus contribute a very large factor to the stability of a protein when $[L] \gg K_d$. Application of this principle worked well for the F219L mutant of bacteriorhodopsin, which was stabilized by the addition of excess retinal, thus allowing its crystallization in hydrated MO. Unfortunately, the same principle also works in reverse when (1) ligands (possibly monoglycerides?) bind to the denatured protein more tightly than to the native protein, or (2) a stabilizing ligand is itself highly soluble in the CBG medium, effectively increasing the value of K_d for the ligand.

FIGURE 9 Illustration of the use of microtiter plates for large-scale, rapid screening of crystallization conditions. (a) Aliquots of gel, as small as 0.2 μL, are first dispensed at the bottom of individual wells, and these are overlaid by any desired solution from a matrix of buffers and precipitants. The gel must not be exposed to air for too long before delivery of the screening solution, or evaporation of water will lead to a phase transition to a form other than the isotropic cubic phase. After all wells have been set up, the entire plate is sealed with ClearSeal tape in order to prevent long-term evaporation of water from the individual wells. (b) Image of one of the wells in the microtiter plate showing a thin film of gel that was accidentally smeared at the bottom of the well during delivery of the main aliquot of gel. (c) Enlarged view of the boxed area shown in panel (b), showing that crystallization can be screened in exceedingly small volumes of gel.

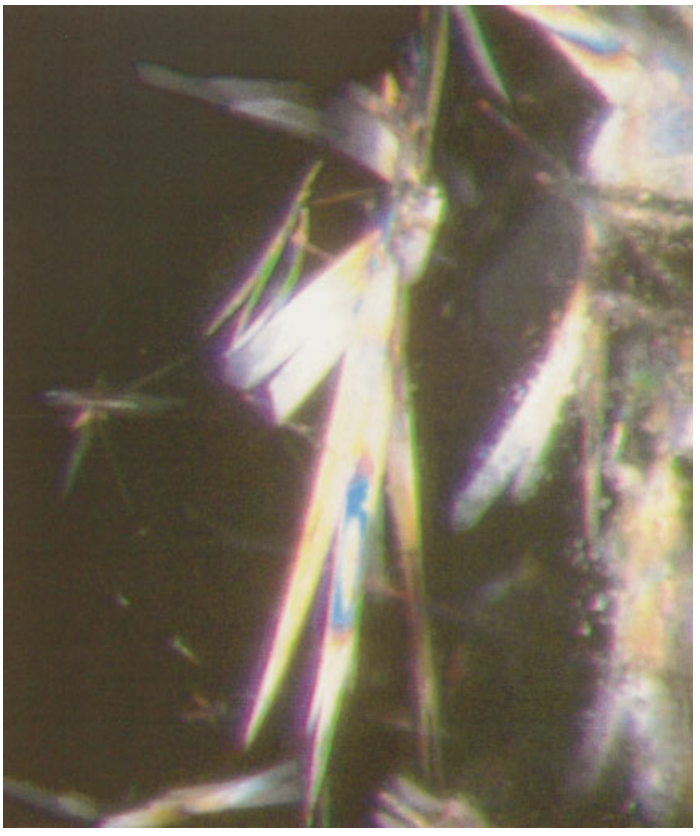


FIGURE 10 Light microscope image of lath-like crystals of mono-olein that sometimes grow within CBG setups. Identical crystals have been observed in the course of attempts to crystallize a number of different membrane proteins.

Inclusion of an inhibitor or other ligand that binds preferentially to the native state is therefore a strategy that is always to be recommended in any screen of crystallization conditions. The benefit of including inhibitors and other ligands is increased even further if they are selective for a unique functional (conformational) substate. In this case, the importance is not just to stabilize the native state of the protein but also to drive the protein into a structurally homogenous population.

The Halobacterial rhodopsins may also be different from other membrane proteins for which crystallization trials have been attempted with CBGs, in that a significant amount of their membrane lipids "copurify" with the detergent-solubilized protein. The purple membrane lipids are, of course, automatically included when the crystallization setup uses whole, solubilized membrane. Even when solubilized bR is purified by column chromatography, however, a substantial amount of lipid still copurifies with the proteins, as is shown by the fact that Halobacterial lipid molecules are included in the protein crystals that subsequently grow from the CBG. The importance of native membrane lipids is established even more clearly in the case of Halobacterial sensory rhodopsin II, where Halobacterial lipids had to be added in order to grow protein crystals from the MO gel.[11,12]

The inclusion of membrane lipids can be expected to improve the success of crystallization from CBGs for several reasons. Membrane proteins are already stabilized by the addition of lipids while still in the detergent-solubilized state, of course. In addition, our observation of accelerated growth of wild-type bacteriorhodopsin crystals illustrates that crystal growth can sometimes be facilitated by the addition of excess lipid. In other cases we have even observed that the crystal space group will be influenced by the presence of excess lipid (i.e., the formation of hexagonal crystals of bacteriorhodopsin in monopalmitin). Finally, a fundamental rationale for including excess membrane lipid as a "guest" in the CBG "host" is the empirical observation that the lipids that form the bilayer gel are themselves not necessarily good ones to serve as a kind of mortar, or filler, between the protein "bricks" within the plane of a 2D crystal. The need to provide some type of "mortar" is self-evident when one dis-

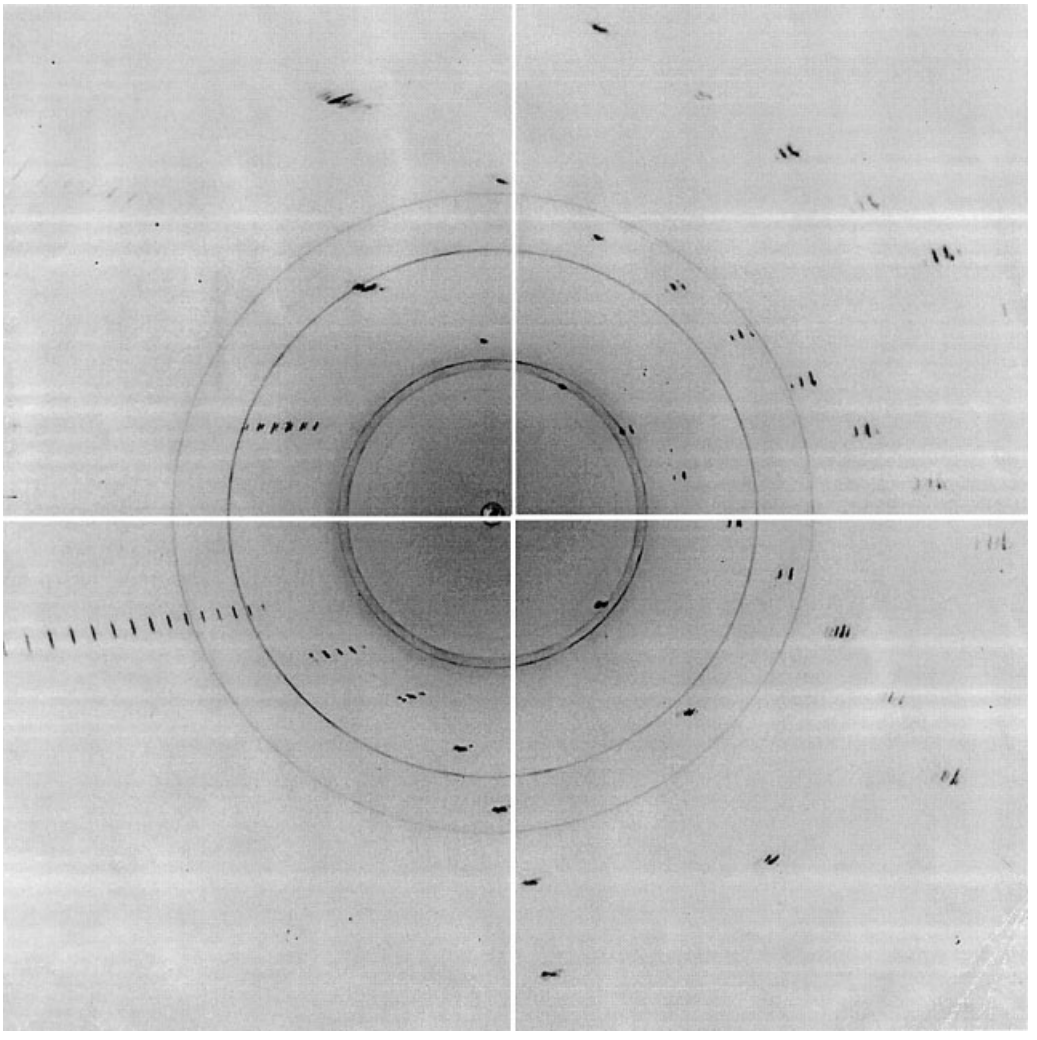


FIGURE 11 Example of an x-ray diffraction pattern recorded from a crystal like the one shown in Figure 10. The presence of only one large unit-cell spacing demonstrates that these are not protein crystals.

cusses the process of formation of 2D crystals that are suitable for electron microscopy, and the same must be true for the growth of coherently stacked layers of 2D crystals.

One of the dangers that may be associated with the addition of membrane lipids to the CBG medium is that the guest lipid may destabilize the host gel. The $pn\bar{3}m$ phase of MO, for example, can tolerate only far less than a 1:4 ratio of guest lipid. Thus it is reasonable to suppose that the molecular environment within the CBG may already be perturbed by the addition of even lower amounts of guest lipids.

Our recent experience with the monopalmitin CBG indicates that this system may open the possibility of doing crystallization screens at lower temperatures than can be achieved with the mono-olein gel. The ability to carry out crystallization screens at lower temperatures would be a significant advantage for proteins that are more stable at lower temperature. Monopalmitin will clearly have other effects on the crystallization process as well. We hypothesized, for example, that the preference to form rhombic crystals of bacteriorhodopsin might be due to the MP gel acting as a more aggressive "solvent" for the Halobacterial lipids, thereby denying the protein access to the amount of membrane lipid that it needs in order to form hexagonal crystals. The validity of this conjecture is supported by the outcome of crystallization trials that were conducted at room temperature, in which hexagonal crystals were able to grow from the monopalmitin gel when excess Halobacterial lipid was added to the system.

Our experience with bacteriorhodopsin indicates that the dimensionality of parameter space that is

associated with crystallization of membrane proteins from CBGs will be significantly larger than is the case for soluble proteins. Crystallization from a CBG will continue to involve all of the familiar dimensions such as temperature, pH, ionic strength, and the use of water-soluble precipitants. This is expected to be true because the formation of coherently ordered layers of a 3D crystal is based on the same type of protein–protein interactions that are needed to form crystals of a soluble protein. In addition, however, new dimensions of parameter space will arise because of the need to form favorable, two-dimensional protein–protein contacts within "the plane of the lipid bilayer." Examples of what these new parameters may involve are the type of membrane lipids that are added as guests within the CBG, the type of host lipid that is used to form the hydrated gel, the type of detergent that is used to solubilized and purify the protein, and possibly the use of other lipid-phase precipitants that could play a role similar to the more familiar aqueous-phase precipitants.

The recent success that we have had in using the isotropic monopalmitin gel to get well-diffracting crystals of bR both at room temperature (by addition of excess lipid) and at 4°C suggests that it will also be important to vary the type of monoglyceride used to form the CBG as an additional dimension in parameter space. Phosphatidylethanols are another class of gel-forming lipids that need to be investigated, although in this case the extreme conditions needed to form such gels suggests that it may first be necessary for new methods to be developed by which the membrane protein is reconstituted into the hydrated gel.

The high dimensionality of parameter space involved in crystallization from CBG media demands that one adopt high-throughput technologies to set up the screening matrices that are required. Methods that use standard microtiter plates to set up the crystallization trials represent one approach that achieves more efficient use of protein and much faster throughput in screening large matrices of crystallization conditions. These methods could be subsequently adapted for setting up screens with a crystallization robot.

The use of CBG media has the potential to scale down the sample volumes to the level of 10 nL or less. As one can see from the example that is shown in Figure 9c, crystallization of bacteriorhodopsin occurs even in a very thin film of gel, "smeared" on the surface of a microtiter well. Delivery of gel volumes as small as 10 nL would require reengineering of the delivery technology, of course, but doing so represents no significant conceptual difficulties.

The use of 10 nL sample volumes has the welcome advantage of partially compensating for the low levels of expression that are commonly encountered for recombinant membrane proteins, and the equally low levels of membrane proteins that are often available from natural sources. Our experience with bacteriorhodopsin has been that crystallization can be successful at protein concentrations as low as 4 mg/mL (in the gel), but that one is less likely to miss a promising condition if the protein concentration is 10 mg/mL. Even at the higher protein concentration one would therefore use only 0.1 μg of protein per screening condition, and up to 1000 conditions could be screened with only 100 μg of purified protein. The reduced amount of protein that may ultimately be practical to use as a result of further development of the CBG technique would thus be a real advantage in the case of membrane proteins.

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